

Frequency, Diversity, and Activity of 2,4-Diacetylphloroglucinol-Producing Fluorescent *Pseudomonas* spp. in Dutch Take-all Decline Soils

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ABSTRACT

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Natural suppressiveness of soils to take-all disease of wheat, referred to as take-all decline (TAD), occurs worldwide. It has been postulated that different microbial genera and mechanisms are responsible for TAD in soils from different geographical regions. In growth chamber experiments, we demonstrated that fluorescent *Pseudomonas* spp. that produce the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) play a key role in the natural suppressiveness of two Dutch TAD soils. First, 2,4-DAPG-producing fluorescent *Pseudomonas* spp. were present on roots of wheat grown in both of the TAD soils at densities at or above the threshold density required to control take-all of wheat; in a complementary take-all conducive soil, population densities of 2,4-DAPG-producing *Pseudomonas* spp. were below this threshold level. Second, introduction of 2,4-DAPG-producing strain SSB17, a representative of the dominant geno-

typic group found in the Dutch TAD soils, into the take-all conducive soil at population densities similar to the densities of indigenous 2,4-DAPG producers found in TAD soils provided control of take-all similar to that observed in the TAD soil. Third, a mutant of strain SSB17 deficient in 2,4-DAPG production was not able to control take-all of wheat, indicating that 2,4-DAPG is a key determinant in take-all suppression. These results show that in addition to the physicochemically different TAD soils from Washington State, 2,4-DAPG-producing fluorescent *Pseudomonas* spp. are also a key component of the natural suppressiveness found in Dutch TAD soils. Furthermore, it is the first time since the initial studies of Gerlagh (1968) that at least part of the mechanisms and microorganisms that operate in Dutch TAD soils are identified. Although quantitatively similar, the genotypic composition of 2,4-DAPG-producing *Pseudomonas* spp. varied between the Dutch TAD soils and the TAD soils from Washington State.

Additional keywords: microbial ecology, rhizosphere competence, suppressive soils.

Take-all, caused by the fungus *Gaeumannomyces graminis* var. *tritici*, is an important root disease of wheat worldwide (2). Although wheat is particularly susceptible to the take-all fungus, many other Gramineae can be infected (34). Take-all decline (TAD) is defined as the spontaneous decrease in the incidence and severity of take-all that occurs with monoculture of wheat or other susceptible host crops after one or more severe outbreaks of the disease (10,19,34). Soils in the state of TAD are naturally suppressive to the take-all fungus. The biological basis of the specific suppression associated with TAD has been demonstrated in a series of experiments: suppressiveness is eliminated by treating the soil with moist heat (pasteurization, 60°C for 30 min), methyl bromide, or chloropicrin, and is transferable by adding small amounts of TAD soil to raw conducive, fumigated or pasteurized soil (10,11, 28). The mechanisms responsible for TAD involve microbiological changes in the bulk soil or rhizosphere environment resulting in suppression of the pathogen (9). Repeated introduction of *G. graminis* var. *tritici* into soil also induces suppression of take-all (17,42,45), but the relation of this form of suppressiveness to that of TAD is not clear.

The occurrence of TAD throughout the world is remarkable in view of the broad range of soil types, climates, and agronomic conditions under which wheat is cultivated (34). Field studies

have clearly indicated that the development of TAD follows a consistent pattern everywhere requiring the presence of three components: monoculture of a take-all susceptible host, the presence of *G. graminis* var. *tritici*, and at least one occurrence of severe disease. Factors such as soil type and previous cropping history seem only to modulate the extent and speed of development of TAD (34). Previous work on TAD has suggested that different microbial genera and mechanisms are responsible for suppressiveness in soils from different geographical regions. First, TAD develops in multiple agroecosystems (6,34). Second, the length of time for TAD to develop varies among fields and the crop grown in monoculture (34). Finally, antagonists from many different taxonomic groups have been isolated from TAD soils and, when introduced into take-all conducive soils, provide a certain level of control of take-all (1,13,19,41,44). Despite the wide range of microorganisms implicated in TAD, however, several of them do not fit one or more of the biological properties of TAD (9). For example, the sensitivity of the TAD factors to pasteurization with moist heat (60°C, 30 min) rules out the involvement of heat-resistant spore-forming bacteria like *Bacillus* spp. and probably also actinomycetes (10).

Among the antagonistic bacteria, the fluorescent *Pseudomonas* spp. have been implicated in TAD soils throughout the world (11,28,32,36,38,41). Characteristics that suggest involvement of pseudomonads in TAD include the following: they are well adapted to the rhizosphere environment and produce a variety of secondary metabolites, including antibiotics and siderophores, that inhibit the growth of *G. graminis* var. *tritici* (38,41), their population densities

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increase considerably on roots with take-all lesions (4,6,8,32,36), and their populations are substantially diminished by soil pasteurization, which also eliminates specific suppression (10,28). Recent studies demonstrated that fluorescent *Pseudomonas* spp. producing the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) play a key role in the suppressiveness of TAD soils in Washington State (27,28,30). Using specific primers and probes directed against sequences within the biosynthetic locus of 2,4-DAPG have clearly indicated that fluorescent *Pseudomonas* spp. producing this antifungal metabolite are highly enriched in TAD soils from Washington State (30). Substantial reduction of populations of 2,4-DAPG producers resulted in loss of suppressiveness (28). When a 2,4-DAPG-producing strain was introduced via seed treatment into a take-all conducive soil, it provided control of take-all of wheat to levels similar to that obtained in the complementary TAD soil (28).

One key question has been whether 2,4-DAPG-producing *Pseudomonas* spp. also play a key role in the specific suppression found in TAD soils from other geographical regions. *Pseudomonas* spp. harboring *phlD* (*phlD*⁺), one of the key genes in the biosynthesis of 2,4-DAPG, were found on roots of wheat grown in other soils with a history of wheat monoculture (24), however, the level of take-all suppressiveness of these soils and the role of 2,4-DAPG in take-all suppression were not addressed. In this study, we determined the frequency, diversity, and activity of 2,4-DAPG-producing *Pseudomonas* spp. found in Dutch polder soils with a history of wheat monoculture. Dutch polder soils were among the first TAD soils reported (17), however, the exact mechanisms operating in these soils have not been elucidated. Phenotypic and genotypic analyses were performed to characterize 2,4-DAPG-producing *Pseudomonas* isolates obtained from Dutch soils. The biocontrol efficacy and rhizosphere competence of *Pseudomonas fluorescens* SSB17, a representative of the major genotypic group of 2,4-DAPG-producers found in a Dutch soil, were compared with that of Q8r1-96, a representative of the major genotypic group of 2,4-DAPG-producers found in TAD soils from Washington State (29). The role of 2,4-DAPG in control of take-all and in rhizosphere competence was determined for both strains by generating mutants deficient in 2,4-DAPG production. Finally, we determined whether populations of 2,4-DAPG-producing *Pseudomonas* spp. are specifically enriched upon infection of wheat roots by the take-all fungus.

MATERIALS AND METHODS

Microorganisms. Naturally occurring populations of fluorescent *Pseudomonas* spp. were isolated from the wheat rhizosphere on King's medium B (KMB) agar supplemented with chloramphenicol (13 µg ml⁻¹), ampicillin (40 µg ml⁻¹), and cycloheximide (100 µg ml⁻¹) [KMB⁺] (35). All *Pseudomonas* strains described in this study, including SSB17 (this study), Q8r1-96 (29), Pf5 and CHA0 (21), were grown on KMB. Spontaneous rifampicin-resistant derivatives of SSB17 and Q8r1-96 were selected on KMB supplemented with rifampicin (100 µg ml⁻¹) and included in the biological control and rhizosphere competence assays. In these bioassays, background levels of indigenous, rifampicin-resistant microorganisms were below detection limit. Strains 9H4 and 4C5 are 2,4-DAPG-deficient mutants derived from strain SSB17 and

Q8r1-96, respectively. 2,4-DAPG-deficient mutants are resistant to rifampicin (100 µg ml⁻¹) and kanamycin (100 µg ml⁻¹). *Escherichia coli* strain S17 λ *pir* was obtained from L. S. Thomashow (USDA-ARS, WA) and contained the mini-Tn5lacZ element in plasmid pUT (14). S17 λ *pir* was grown in liquid Luria Bertani (LB) amended with kanamycin (25 µg ml⁻¹). All bacterial strains were stored at -80°C in LB or KMB broth supplemented with 40% (vol/vol) glycerol.

Strain R3-111a-1 of *G. graminis* var. *tritici* (the causal agent of take-all) was originally isolated from wheat grown in a soil near Moses Lake, Washington State. Strain C-1 of *Rhizoctonia solani* anastomosis group (AG)-8 (the causal agent of Rhizoctonia root rot) was obtained from the collection maintained by the USDA-ARS, Pullman, WA. Strain CBS 219.65 of *Pythium ultimum* var. *sporangiferum* (the causal agent of Pythium root rot) was obtained from the Dutch collection of microorganisms (CBS, Baarn, The Netherlands). *G. graminis* var. *tritici*, *R. solani*, and *Pythium ultimum* var. *sporangiferum* were routinely grown on potato dextrose agar (PDA; Oxoid Ltd., Basingstoke, Hampshire, England). Mycelial plugs were immersed in sterile mineral oil and stored at 15°C.

Soils. Soils CB, SV, and SSB were obtained in December 1997, 1999, and 2001 from agricultural polder fields in Woensdrecht, The Netherlands. Woensdrecht is located in the southwest of the Netherlands, 10 km from the city Bergen op Zoom. In 1997, the SV and SSB soil had been continuously cropped to wheat for 14 and 27 years, respectively; the CB soil was grown to wheat and sugarbeet in a 1:2-year rotation scheme. Soils were collected from the top 50 cm of the soil profile, air dried for a week, and passed through a 0.5-cm mesh screen prior to use. Chemical and physical properties of the soils used in this study are listed in Table 1.

Natural suppressiveness of soils to *G. graminis* var. *tritici* and *R. solani*. Soils were amended with 0.1 to 0.4% (wt/wt) of an oat grain inoculum of *G. graminis* var. *tritici* strain R3-111a-1 (particle size 0.25 to 0.50 mm) (28); sterilized oat grain inoculum was used as a control. The inoculum used in the experiments was obtained from different batches and therefore the concentration varied between experiments to ensure similar disease levels. Wheat seeds (cv. Bussard) were sown in PVC pots (8 cm high, 7 cm wide) containing 200 g of soil and covered with a 1-cm layer of soil without inoculum. Plants were grown for 4 weeks in climate chambers at 15°C with a 12-h photoperiod. Twice a week, plants received 50 ml of 1/3-strength Hoagland's solution (macroelements only). Each treatment had five replicates with approximately 12 plants per replicate. Eight randomly selected plants were harvested, their root systems washed and the severity of take-all determined on a 0-to-8 scale, where 0 indicates no disease and 8 indicates a dead plant (28). Similar to assays performed with *G. graminis* var. *tritici*, suppressiveness of the soils to *R. solani* was assessed by introducing 0.5% (wt/wt) of a whole millet grain inoculum of *R. solani* AG-8 3 days prior to planting the wheat seeds; sterilized millet grain was used as a control. To assess the level of suppressiveness to *R. solani* infections, roots of eight randomly selected plants were harvested and washed, and root dry weight determined. From each replicate, three to four randomly selected plants were collected for isolation of naturally occurring pseudomonads from the rhizosphere.

TABLE 1. Cropping history and physicochemical properties of the soils used in this study

Soil	Cropping history	Wheat cultivar	pH ^y	Content (µg per g of soil)					Content (%)			
				NO ₃ -N	NH ₄ -N	Na	P	K	OM ^z	Sand	Silt	Clay
CB	Rotation wheat/sugar beet	Bussard	7.6	3	2	14	1	74	4.4	49	12	27
SV	14 years continuous wheat	Monopol	7.4	4	2	10	2	74	4.4	52	9	27
SSB	27 years continuous wheat	Bussard	7.5	3	2	9	2	89	4.4	50	10	28

^y pH was determined after extraction with CaCl₂.

^z Organic matter.

Isolation and enumeration of *phlD*+ fluorescent *Pseudomonas* spp. After 4 weeks of plant growth, three to four plants of each replicate were harvested and loosely adhering soil was removed from the root system. Naturally occurring fluorescent *Pseudomonas* spp. were isolated from uninfected wheat roots and roots infected by *G. graminis* var. *tritici* or *R. solani*. From the infected roots, both nonlesioned and lesioned root sections were selected. Root samples of 0.2 to 1 g fresh weight were vortexed for 1 min and sonicated for 1 min in an ultrasonic cleaner (Branson 12; Branson Ultrasonics Corp., Geneva, Switzerland). Root suspensions were diluted and plated onto KMB⁺ and incubated at 25°C for 48 h. Population densities of *phlD*+ *Pseudomonas* spp., a key gene in the biosynthesis of 2,4-DAPG, were determined by colony hybridization followed by polymerase chain reaction (PCR) (27). *phlD*+ colonies were purified and stored at -80°C for further analyses.

Random amplified polymorphic DNA analysis. Random amplified polymorphic DNA (RAPD) analysis of *phlD*+ isolates was performed with primers M13, M12, and D7. These primers were selected by Keel et al. (21) among 64 other random primers based on the distinct and consistent banding patterns of the polymorphic markers produced. PCR was carried out in a 25- μ l reaction mixture as described previously (30). PCR amplifications were carried out in a thermocycler (PTC-200; MJ Research Inc., Watertown, MA). Samples (10 to 15 μ l) of the PCR products were separated on 2% agarose gels in 1 \times TBE buffer (90 mM Tris-borate and 2 mM EDTA [pH 8.0]) at 80 V for 3 h. Gels were stained with ethidium bromide and bands were visualized on a UV transilluminator. RAPD analysis was repeated at least three times for each primer. The presence or absence of bands that were consistently generated by RAPD analysis (1 for the presence and 0 for the absence of a particular sized band in the gel) was used to calculate the pairwise coefficients of similarity (Nei-Li distances). Cluster analysis with the neighbor joining method and bootstrap analysis were performed with the program FreeTree (18). The dendrogram was edited and visualized in TREEVIEW (26). Data from the RAPD analyses were used to calculate the Shannon-Weaver's diversity index, using the formula $H = -\sum [p_i \cdot \ln(p_i)]$, where p_i is the proportion of each RAPD group (based on 100% similarity) in relation to the total number of RAPD groups per soil.

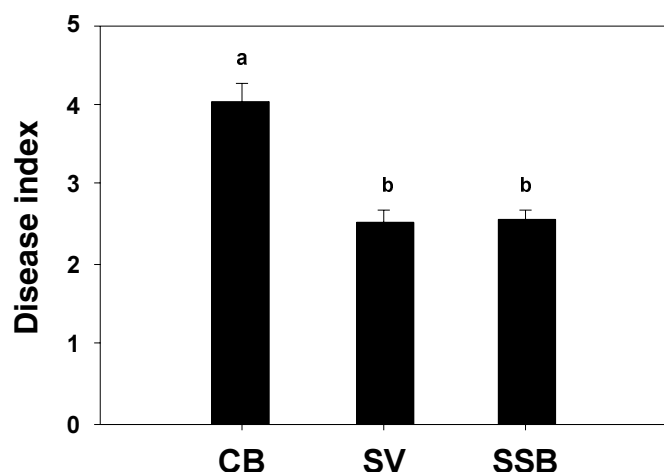


Fig. 1. Natural suppressiveness of soils CB, SV, and SSB to *Gaeumannomyces graminis* var. *tritici*. Soils were amended with 0.1% of an oat grain inoculum of *G. graminis* var. *tritici*. Plants were grown for 1 month under controlled conditions and disease caused by *G. graminis* var. *tritici* was scored on a 0-to-8 scale, where 0 indicates no disease and 8 indicates dead plant. Means of five replicates are shown. Means with the same letter are not statistically different according to Tukey's studentized range test ($P = 0.05$). Error bars represent the standard error of the mean. The experiment was repeated four times and representative results are shown.

Plate inhibition assays. *PhlD*+ *Pseudomonas* isolates obtained from roots of wheat grown in Dutch agricultural soils were inoculated at the edges of 9-cm plates containing 1/5-strength PDA (initial pH = 6.5) and incubated for 2 days at 25°C. Mycelial plugs (5-mm-diameter) of *G. graminis* var. *tritici*, *R. solani*, and *Pythium ultimum* var. *sporangiferum* were transferred to the center of the plate. Plates were incubated at 25°C and the radial growth of the fungus toward the bacterial isolate was measured after 3 to 5 days and compared with the control (fungus only). For each isolate, growth inhibition of both *G. graminis* var. *tritici* and *R. solani* was determined in duplicate. Data from plate inhibition assays were used to construct similarity matrices, where a hierarchical cluster analysis was performed by the single linkage method (12).

Tn5-mutagenesis. 2,4-DAPG-deficient mutants of spontaneous rifampicin-resistant derivatives of *Pseudomonas fluorescens* strains SSB17 and Q8r1-96 were obtained by biparental mating with *E. coli* strain S17 λ *pir* harboring the mini-Tn5*lacZ* element in plasmid pUT, according to protocols described by Sambrook and Russel (31). Transformants were selected on KMB supplemented with rifampicin and kanamycin and subsequently transferred to 98-well microtiter plates containing KMB broth. Transformants unable to produce a red pigment, which is not 2,4-DAPG, but is characteristic for 2,4-DAPG-producing *Pseudomonas* strains (3), were selected after 1 week of growth at 25°C. High-performance liquid chromatography (HPLC) was used to confirm the inability of these transformants to produce 2,4-DAPG (5). Plate inhibition assays with *G. graminis* var. *tritici*, *R. solani*, and *Pythium ultimum* var. *sporangiferum* were used to further confirm the inability of mutant strains to inhibit these pathogens in vitro. RAPD analysis with primers M13, M12, and D7 was performed to confirm strain integrity. Southern blot analysis was used to determine the number of copies of the Tn5*lacZ* element in the 2,4-DAPG-deficient mutants.

Southern analysis and colony hybridizations. For Southern blot analysis, genomic DNA of wild type and mutant strains was extracted with the DNA Wizard Kit (Promega, Leiden, The Netherlands). Samples containing 2.0 μ g of DNA were digested with 5 units of *KpnI* and *EcoRI* (Promega), two enzymes for which restriction sites are not present in the kanamycin gene of the mini-Tn5*lacZ* element (14). Restrictions were performed in a total volume of 100 μ l at 37°C for 12 h. Digested DNA was precipitated with 4 M LiCl, washed with 70% ethanol, dissolved in 15 μ l of sterile distilled water, and separated on 1% agarose gels in 1 \times TBE. DNA and colony transfer to Hybond N⁺ nylon membranes (Amersham Pharmacia Biotech, Roosendaal, Netherlands) were performed according to standard methods (31). Membrane washes and hybridizations were performed following standard procedures (31). High-stringent conditions comprised prehybridization for 1.5 h at 65°C, hybridization for 12 h at 65°C, membrane washing twice for 5 min each with 2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate (SDS) at room temperature, and membrane washing twice each for 30 min with 0.1 \times SSC-0.1% SDS at 65°C. A 575-bp probe, specific for the kanamycin gene contained within the Tn5*lacZ* element, was obtained by direct PCR digoxigenin (DIG) labeling (Roche Corp., Basel, Switzerland) of the fragment using primers KM1 (5'-CCCGATGCGCCAGAGTTGTT) and KM2 (5'-TCACCGAGGCAGTTCATAGG).

Treatment of seeds with *Pseudomonas*. For bioassays and rhizosphere competence studies, wheat seeds (cv. Bussard) were coated with SSB17, Q8r1-96, or their 2,4-DAPG-deficient mutants in 1% methylcellulose to final densities of approximately 10³, 10⁴, and 10⁶ CFU per seed; seeds treated with 1% methylcellulose served as a control. Population densities of introduced *Pseudomonas* spp. strains SSB17, Q8r1-96, and their 2,4-DAPG-deficient mutants were assessed on uninfected wheat roots and on roots infected by *G. graminis* var. *tritici*.

Statistical analysis. Prior to analysis of variance (ANOVA), data from population counts were log₁₀-transformed and disease index data were ranked. Root dry weight data were analyzed directly by ANOVA followed by Tukey's studentized range test, after certifying normal distribution and homogeneity of variances (SAS Institute, Cary, NC). Data from bacterial population counts on *G. graminis* var. *tritici*- and *R. solani*-lesioned roots that did not present normal distribution after transformations log₁₀ (CFU) were analyzed by the one-way nonparametric Kruskal-Wallis test and comparison of means of two treatments was performed by Wilcoxon's two sample test. Data from population increases were also analyzed by the one-way nonparametric Kruskal-Wallis test followed by Wilcoxon's two sample test. Data from rhizosphere competence assays were analyzed by nonlinear regression analysis to determine the relationship between the initial density of strains SSB17 and Q8r1-96 on seeds and their final density on roots of 1-month-old wheat plants. The equation used was $Y = a \times X/(b + X)$, where Y represents the final density (log CFU g⁻¹ root), X represents the initial density (log CFU per seed), a represents the maximum final density, and b represents the initial density necessary to reach half of the maximal final density (29). All experiments were performed at least twice and representative results are shown.

RESULTS

Natural suppressiveness of soils to *G. graminis* var. *tritici*. Characteristics of soils CB, SV, and SSB are shown in Table 1. Physical and chemical properties of these soils differ from TAD soils from Washington State (30). Major differences are that the Dutch soils have lower levels of nitrate, phosphorus, potassium, and silt content, and higher levels of organic matter and clay content compared with the four soils from Washington State. The three Dutch soils were tested in bioassays for suppressiveness to *G. graminis* var. *tritici*. When these soils were inoculated with *G. graminis* var. *tritici*, the disease index was significantly lower on roots of plants grown in SV and SSB soils compared with plants grown in CB soil (Fig. 1). No take-all symptoms were found on roots of wheat grown in soils not inoculated with *G. graminis* var. *tritici* (data not shown). Very similar levels of take-all suppressiveness were described for the Washington State soils (28). Based on these results and the occurrence of long-term wheat monoculture in the SV and SSB soils, they were classified as TAD soils, whereas CB soil was regarded as a *G. graminis* var. *tritici*-conductive soil. When inoculated with *R. solani* AG-8, root dry weights were significantly reduced in all three soils by approximately 40% compared with the healthy controls. No significant differences in reduction of root biomass were observed between the two Dutch TAD soils and the take-all conducive CB soil.

Frequency and diversity of 2,4-DAPG-producing *Pseudomonas* spp. in Dutch TAD soils. In CB soil, wheat rhizosphere population densities of *phlD*+ *Pseudomonas* spp. were relatively low and ranged from less than 10⁴ to 2.7 × 10⁴ CFU/g of root (Table 2). In the SV and SSB soils, populations of *phlD*+

fluorescent *Pseudomonas* spp. ranged from 5.1 × 10⁴ to 1.4 × 10⁶ CFU/g of root (Table 2). These populations represented approximately 5 to 14% of the total population of fluorescent pseudomonads isolated from wheat roots and were, on average, 5- to 52-fold higher than populations of *phlD*+ fluorescent *Pseudomonas* spp. found on roots of wheat grown in the CB soil. These results demonstrate a correlation between relatively high populations of *phlD*+ fluorescent *Pseudomonas* spp. and suppressiveness to take-all of wheat.

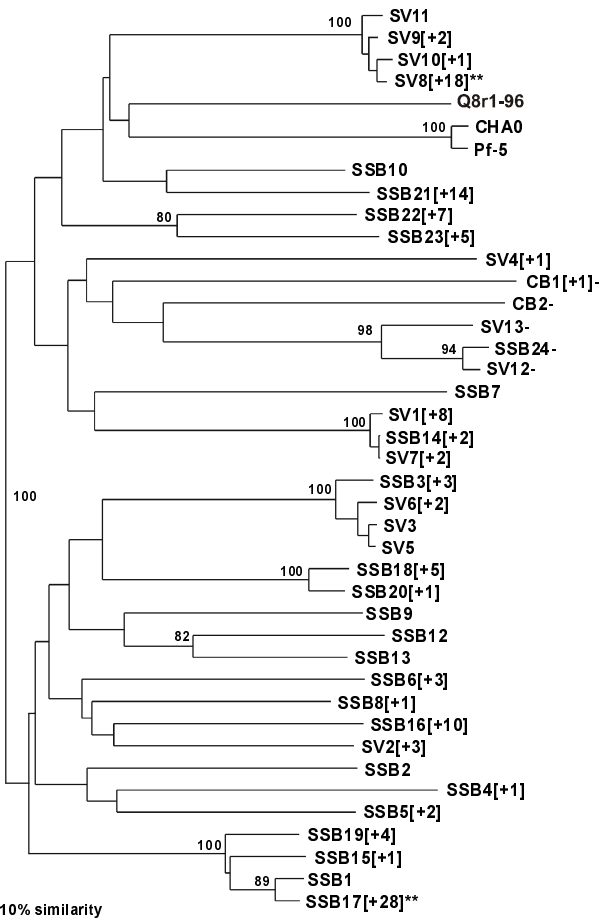


Fig. 2. Dendrogram of *phlD*+ fluorescent *Pseudomonas* spp. strains based on random amplified polymorphic DNA (RAPD) markers. The pairwise coefficients of similarity (Nei/Li distances) were clustered using the neighbor-joining method. On roots of wheat grown in soil, SV, 11 (SV1 to SV11) different genotypic groups of *phlD*+ isolates were found on the basis of 100% similarity. In soil SSB, 23 (SSB1 to SSB23) different groups were found. Each branch represents a unique genotypic group. Isolates followed by – are *phlD*– fluorescent *Pseudomonas* spp. Numbers between brackets represent the additional number of isolates in the RAPD group. Asterisks indicate the major genotypic groups in SV and SSB soils. Bootstrap values were calculated for 1,000 resampled data sets and only values equal or higher than 80% are shown.

TABLE 2. Frequency of *phlD*+ fluorescent *Pseudomonas* spp. in the wheat rhizosphere

Soil	First experiment			Second experiment		
	Total fluorescent <i>Pseudomonas</i> spp. ^x	<i>phlD</i> + fluorescent <i>Pseudomonas</i> spp. ^y	% <i>phlD</i> + fluorescent <i>Pseudomonas</i> spp. ^z	Total fluorescent <i>Pseudomonas</i> spp. ^x	<i>phlD</i> + fluorescent <i>Pseudomonas</i> spp. ^y	% <i>phlD</i> + fluorescent <i>Pseudomonas</i> spp. ^z
CB	2.7 × 10 ⁵	<10 ⁴	<0.1	1.2 × 10 ⁶	2.7 × 10 ⁴	2.3
SV	5.7 × 10 ⁵	5.1 × 10 ⁴	9.0	4.5 × 10 ⁶	2.1 × 10 ⁵	4.8
SSB	1.1 × 10 ⁶	1.1 × 10 ⁵	14.0	1.1 × 10 ⁷	1.0 × 10 ⁶	12.7

^x Total population of fluorescent *Pseudomonas* spp. (CFU/g of root). Mean values of five replicates are given.
^y Population of fluorescent *Pseudomonas* spp. harboring the *phlD* gene (CFU/g of root). Mean values of five replicates are given.
^z Percentage of fluorescent *Pseudomonas* spp. harboring the *phlD* gene in relation to the total fluorescent *Pseudomonas* population. Mean values of five replicates are given.

Analysis of the genotypic diversity of the isolated *phlD*+ fluorescent *Pseudomonas* spp. by RAPD analysis with primers M13, M12, and D7 generated a total of 58, 56, and 48 consistent RAPD markers, respectively, ranging in size from 190 to

TABLE 3. Clustering of *phlD*+ fluorescent *Pseudomonas* spp. by random amplified polymorphic DNA (RAPD) analysis[†]

Origin/type of isolate	No. of isolates	RAPD groups ^u	Major groups (%) ^v	Shanon-Weaver index ^w
SV soil	48	11	39.6 (SV8)	32.2
SSB soil	113	23	25.7 (SSB17)	84.2
SV and SSB soils ^x	6	1
Non-2,4-DAPG producers ^y	6	5
Reference strains ^z	3	3

[†] *phlD*+ isolates were obtained from roots of wheat grown in SV and SSB soils.

^u RAPD groups were defined with primers D7, M12, and M13 on the basis of 100% similarity.

^v Major group refers to the percentage of the total number of *phlD*+ fluorescent *Pseudomonas* represented by a single dominant genotype.

^w Calculated by using the formula $H = \sum [p_i - \ln(p_i)]$, where p_i is the proportion of each RAPD group (based on 100% similarity) in relation to the total number of RAPD groups per soil.

^x Present in both, SV and SSB soils.

^y *phlD*- isolates include CB1, CB2, CB3, SV12, SV13, and SSB24 and were obtained from roots of wheat grown in CB, SV, and SSB soils.

^z Reference strains include *Pseudomonas fluorescens* strains CHA0, Pf5, and Q8r1-96.

2,070 bp. Reproducibility of the RAPD amplifications was confirmed in three independent experiments and the reliability of the dendrogram topology (Fig. 2) was evaluated by bootstrap analysis using 1,000 resampled data sets. Among a total of 161 isolates of *phlD*+ fluorescent *Pseudomonas* spp. obtained from roots of wheat grown in SV and SSB soil, 33 unique groups were identified and only one RAPD group, represented by isolates SSB14 and SV7, was found in both soils (Fig. 2; Table 3). The major genotypic groups found on roots of wheat grown in SV and SSB soils represented on average 40 and 26%, respectively, of the total number of *phlD*+ fluorescent *Pseudomonas* spp. isolates from SV and SSB soils (Table 3). The major genotypic group (SV8) found in SV soil clustered very distantly from the major genotypic group (SSB17) found in SSB soil (Fig. 2). Isolates from SV and SSB soils clustered distantly from 2,4-DAPG-producing reference strains CHA0, Pf5, and Q8r1-96 and from 2,4-DAPG-nonproducing isolates (groups CB1, CB2, SV12, SV13, and SSB24) obtained from CB, SV, and SSB soils (Fig. 2). The Shanon-Weaver's diversity index was higher for populations of *phlD*+ fluorescent *Pseudomonas* spp. isolated from SSB soil than for those from SV soil (Table 3), indicating a higher level of genotypic diversity in SSB soil.

In addition to genotypic clustering by RAPD analysis, data from plate inhibition assays were used to phenotypically group the isolates on the basis of their capacity to inhibit *G. graminis* var. *tritici* and *R. solani*. Based on *G. graminis* var. *tritici* mycelial inhibition, a total of seven clusters were obtained. Five of these

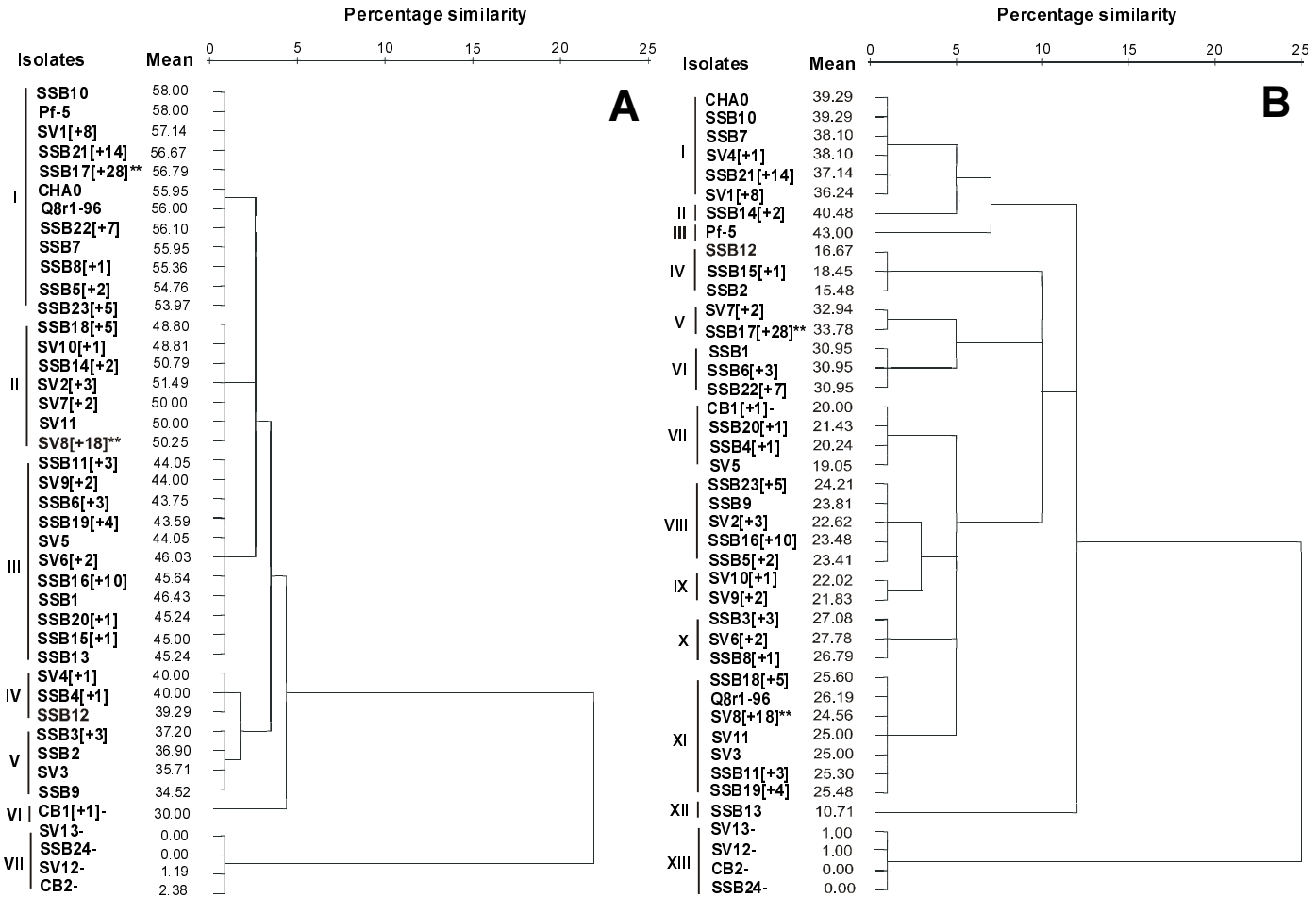


Fig. 3. Cluster analysis of in vitro inhibition of **A**, *Gaeumannomyces graminis* var. *tritici* and **B**, *Rhizoctonia solani* by *phlD*+ fluorescent *Pseudomonas* spp. Hierarchical, single linkage cluster analysis was computed using the mean inhibition of mycelial growth on plates given by a certain *Pseudomonas* isolate when compared with the control (the fungus only). Numbers between brackets represent the number of additional isolates in the cluster. Asterisks indicate the major genotypic groups in SV and SSB soils. Isolates followed by - are *phlD*- fluorescent *Pseudomonas* spp. Mean refers to the mean percentage inhibition relative to the control.

clusters (I to V) contained isolated 2,4-DAPG producers and reference strains Pf5, CHA0, and Q8r1-96, and two clusters (VI and VII) contained *phlD*- isolates that did not produce 2,4-DAPG. Thirteen clusters were obtained based on inhibition of mycelial growth of *R. solani*. Clusters I to XI contained isolated 2,4-DAPG producers and reference strains and clusters XII and XIII contained non-2,4-DAPG producers (Fig. 3). Comparison of the cluster analyses indicates that 2,4-DAPG-producing isolates are more inhibitory in vitro to *G. graminis* var. *tritici* than to *R. solani* (Fig. 3). The mean inhibition of mycelial growth by *phlD*+ fluorescent *Pseudomonas* spp. isolates and 2,4-DAPG-producing reference strains ranged from approximately 35 to 58% for *G. graminis* var. *tritici* and from approximately 11 to 43% for *R. solani*. For 2,4-DAPG nonproducing isolates, inhibition of *G. graminis* var. *tritici* ranged from 0 to 30% and inhibition of *R. solani* ranged from 0 to 20%. Most phenotypic groups contained more than one RAPD group and isolates belonging to the same RAPD group (based on 100% similarity) were always grouped in the same phenotypic group (Figs. 2 and 3). Strain SSB17, which is a representative of the major RAPD group found on roots of wheat grown in SSB soil (Fig. 2; Table 3), clustered in the phenotypic group that was most inhibitory to *G. graminis* var. *tritici* (Fig. 3) and was selected for further studies. Strain Q8r1-96, which represents the major RAPD group among 2,4-DAPG-producing *Pseudomonas* spp. isolated from wheat grown in the Quincy TAD soil from Washington State (29), was included for comparison purposes. Q8r1-96 clustered in the same *G. graminis* var. *tritici*-phenotypic group as SSB17 (Fig. 3), but is genotypically different (Fig. 2).

Role of 2,4-DAPG-producing *Pseudomonas* spp. in Dutch TAD soils. If 2,4-DAPG-producing fluorescent *Pseudomonas* spp. play a determinative role in the natural suppressiveness of Dutch TAD soils, then introduction of a 2,4-DAPG-producing strain into the conducive soil should give suppressiveness to *G. graminis* var. *tritici* to a level similar to that obtained in the complementary TAD soil. Furthermore, if 2,4-DAPG is a key metabolite in suppression of *G. graminis* var. *tritici*, then 2,4-DAPG-deficient mutants should have a reduced ability to suppress take-all of wheat. Tn5 mutagenesis of *Pseudomonas fluorescens* strains SSB17

and Q8r1-96 generated 1,078 and 686 transformants, respectively. For strains SSB17 and Q8r1-96, 4 and 2 transformants, respectively, did not produce the red pigment characteristic of 2,4-DAPG producers. RAPD analysis confirmed strain integrity of all six mutants, and HPLC analysis showed that all six mutants were defective in 2,4-DAPG production. In plate inhibition assays, wild-type strains SSB17 and Q8r1-96 caused clear inhibition of mycelial growth of *G. graminis* var. *tritici*, *R. solani*, and *Pythium ultimum* var. *sporangiferum*, whereas the mutants caused no inhibition of these pathogens (data not shown). Southern blot analysis revealed that all mutants had a single transposon insertion. Mutants 9H4, derived from SSB17, and 4C5, derived from Q8r1-96, were selected for the bioassays.

Strain SSB17 and its 2,4-DAPG-deficient mutant 9H4 were introduced via seed treatment into the take-all conducive CB soil amended with *G. graminis* var. *tritici* inoculum at initial densities of approximately 10^3 CFU per seed. SSB17 and 9H4 established rhizosphere population densities of 5.0×10^6 and 9.0×10^6 CFU/g of root, respectively, after 4 weeks of plant growth. Population densities of the wild type and mutant were not significantly different and were similar to the density of indigenous 2,4-DAPG producers (5.0×10^6 CFU/g of root) found on roots of wheat grown in the SSB soil amended with *G. graminis* var. *tritici* inoculum. Treatment of seeds with strain SSB17 significantly reduced take-all severity and provided control to the same level as found in the SSB TAD soil (Fig. 4A). Mutant 9H4 did not reduce disease severity caused by *G. graminis* var. *tritici* (Fig. 4A). These results provide further evidence that *phlD*+ fluorescent *Pseudomonas* spp. are a key component of the suppressiveness that operates in the SSB TAD soil and that 2,4-DAPG is the major determinant of take-all suppression by strain SSB17. In independent experiments, similar results were found for strain Q8r1-96 and its 2,4-DAPG-deficient mutant 4C5 (Fig. 4B). Although there were no differences between strains SSB17 and Q8r1-96 in their ability to suppress take-all, there was a significant difference in their rhizosphere competence (Fig. 5). In spite of the fact that strain Q8r1-96 was isolated from a different wheat cultivar grown in a physicochemically different soil from another geographical region, it was more rhizosphere competent than strain SSB17.

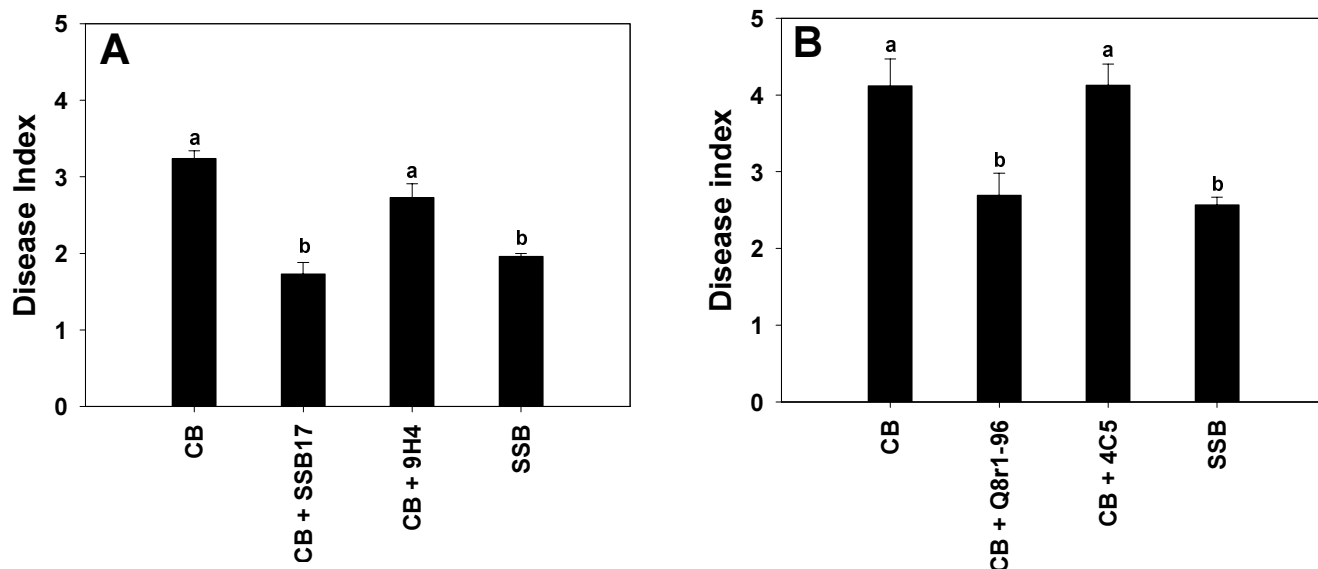


Fig. 4. A, Effect of *Pseudomonas fluorescens* strains SSB17 and its 2,4-diacetylphloroglucinol (2,4-DAPG)-deficient mutant, 9H4, and **B,** Q8r1-96 and its 2,4-DAPG-deficient mutant, 4C5, on infection of wheat by *Gaeumannomyces graminis* var. *tritici*. Seeds treated with bacterial strains at densities of approximately 10^3 CFU per seed were sown in CB soil amended with *G. graminis* var. *tritici* inoculum. Untreated seeds sown in CB and SSB soils served as controls. Plants were grown for 1 month and the severity of take-all was assessed on a 0-to-8 scale. Means of five replicates are shown. Means with the same letter are not statistically different according to Tukey's studentized range test ($P = 0.05$). Error bars represent standard errors of the means. The experiment was repeated two times and representative results are shown.

Dose-response studies showed that strain Q8r1-96 required lower initial densities on seeds than strain SSB17 to reach the same final population on roots (Fig. 5). When comparing dose-response kinetics of the wild-type strains SSB17 and Q8r1-96 with those obtained for their respective 2,4-DAPG-deficient mutants, 9H4 and 4C5, no differences were found (Fig. 5). These results suggest that 2,4-DAPG production does not contribute to the rhizosphere competence of strains SSB17 and Q8r1-96.

Effect of take-all and Rhizoctonia root rot on rhizosphere population densities of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. The influence of *G. graminis* var. *tritici* and *R. solani* infections on wheat root colonization by indigenous and introduced *phlD*+ fluorescent *Pseudomonas* spp. was studied. Population densities of indigenous pseudomonads and *phlD*+ fluorescent *Pseudomonas* spp. were assessed on healthy and *G. graminis* var. *tritici*- or *R. solani*-infected roots of wheat grown in SV and SSB soils, and in more detail on nonlesioned and lesioned root sections of *G. graminis* var. *tritici*- or *R. solani*-infected roots (Tables 4 and 5). When roots of wheat grown in SV and SSB soils were infected with *G. graminis* var. *tritici*, or *R. solani*, densities of *phlD*+ fluorescent *Pseudomonas* spp. on the whole root systems increased significantly by five to eightfold. Identical increases were observed for the total fluorescent *Pseudomonas* population on *G. graminis* var. *tritici*-infected roots and on roots infected with *R. solani*. When population densities of both total and *phlD*+ fluorescent *Pseudomonas* spp. were compared in more detail on lesioned and nonlesioned root sections of infected roots, *G. graminis* var. *tritici* infection led to increases ranging from 4- to 15-fold. Similar increases in population densities of total and *phlD*+ fluorescent *Pseudomonas* spp. were observed in the SV and SSB soils. Population densities of *phlD*+ fluorescent *Pseudomonas* spp. were up to sixfold greater on *R. solani*-lesioned root sections than on nonlesioned root sections. These increases were not significantly different from the increases observed on *G. graminis* var. *tritici*-lesioned root sections for both SV and SSB soils ($P = 0.26$ and 0.47 , respectively).

The effect of *G. graminis* var. *tritici* on root colonization was also determined for *Pseudomonas fluorescens* strains SSB17, Q8r1-96, and their respective 2,4-DAPG-defective mutants, 9H4 and 4C5, all of which were introduced at two different initial densities (Table 6). After 3 weeks of plant growth, rhizosphere

population densities of the wild-type strains and their respective mutants were not significantly different from each other in presence or in absence of *G. graminis* var. *tritici*. Population densities of SSB17 and 9H4 were significantly lower than population densities of Q8r1-96 and 4C5, irrespective of the presence of *G. graminis* var. *tritici*. For both initial densities of the introduced strains, infections of wheat roots by *G. graminis* var. *tritici* did not lead to significant increases in their population densities.

DISCUSSION

The results of this study indicate that 2,4-DAPG-producing *Pseudomonas* spp. play a key role in the suppressiveness of two Dutch TAD soils. First, 2,4-DAPG-producing *Pseudomonas* spp. were present on roots of wheat grown in both TAD soils at densities up to approximately 10^6 CFU/g of root, densities that are equal or higher than the threshold density (10^5 CFU/g of root) of 2,4-DAPG-producing fluorescent *Pseudomonas* strains required to control take-all (28). In the complementary take-all conducive soil, population densities of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. were below this threshold density. Second, introduction of 2,4-DAPG-producing strain SSB17, representative of the dominant genotypic group found in one of the Dutch TAD soils, into the take-all conducive soil at rhizosphere population densities similar to the densities of indigenous 2,4-DAPG-producing fluorescent *Pseudomonas* spp. found in TAD soils, provided control of take-all to the same level as found in the complementary TAD soil. Third, a mutant of SSB17 deficient in 2,4-DAPG production was not able to control take-all of wheat, suggesting that 2,4-DAPG is a key determinant in take-all suppression. Previously, the role of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. in TAD soils had been demonstrated only in Washington State. Gerlagh (17) postulated from his elegant and classic studies of TAD in Dutch polders that specific suppression possibly involves antibiotic production by soil microorganisms. This report identifies for the first time, at least in part, the mechanism and microorganisms that contribute to the suppressiveness of Dutch TAD soils.

The densities of indigenous 2,4-DAPG-producing *Pseudomonas* spp. found on roots of wheat grown in the Dutch TAD soils were quantitatively similar to the densities found in four TAD soils

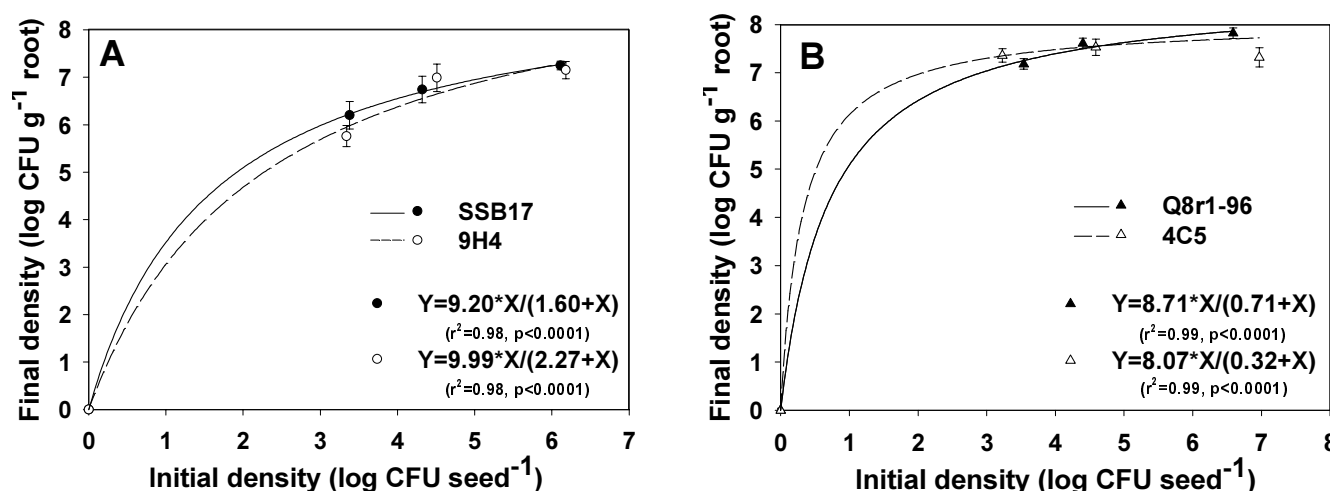


Fig. 5. Relationships between the initial density on seeds (log CFU per seed) and the final densities on roots (log CFU g^{-1} root) for *Pseudomonas fluorescens* strains SSB17, Q8r1-96, and their respective 2,4-diacetylphloroglucinol (2,4-DAPG)-defective mutants, 9H4 and 4C5. Comparison of **A**, the rhizosphere competence of *Pseudomonas fluorescens* strain SSB17 (solid line) and its 2,4-DAPG-deficient mutant 9H4 (dashed line), and **B**, Q8r1-96 (solid line) and its 2,4-DAPG-deficient mutant 4C5 (dashed line). Wheat seeds were treated with each of the strains at densities of approximately 0 , 10^3 , 10^4 , and 10^6 CFU per seed. Plants were grown for 1 month in CB soil and rhizosphere populations of the introduced strains were determined by dilution plating. Means of five replicates are shown. Error bars represent the standard error of the mean. Nonlinear regression was performed to determine the relationship between the initial and final densities of the introduced strains. The equation used was $Y = a \times X / (X + b)$, where Y is the final density, X is the initial density, a is the maximum final density, and b is the initial density necessary to reach half of the maximum final density. The experiment was repeated twice and representative results are shown.

from Washington State (28,30). However, the genotypes of 2,4-DAPG-producers responsible for TAD in the Dutch soils differed from those in Washington State TAD soils (24,29). For example, Raaijmakers and Weller (29) identified 16 RAPD groups among 101 *phlD*+ isolates obtained from roots of wheat grown in Quincy, Washington TAD soil, and one group comprised 50% of the isolates. This genotype also was dominant on roots of wheat grown in Moses Lake and Lind, Washington TAD soils and belonged to BOX-PCR group D as defined by McSpadden-Gardener et al. (24). In the current study, 33 distinct RAPD groups were identified among 161 *phlD*+ isolates from the two Dutch TAD soils, but the SV and SSB soils had different dominant groups that represented 40 and 26%, respectively, of the isolates. All of the Dutch isolates clustered distantly from Q8r1-96, a representative of the dominant *phlD*+ isolates from the three Washington TAD soils. Furthermore, the dominant Dutch isolates corresponded to BOX-PCR groups M and F, which do not occur in Washington TAD soils.

The Shannon-Weaver's index indicated that the diversity among 2,4-DAPG producers was higher in the SSB soil than in the SV soil. This was unexpected because we had hypothesized that the longer history of wheat monoculture in the SSB soil compared with the SV soil would have enriched for a narrower range of genotypes. One explanation for the difference in diversity index between the two soils may be the fact that under field conditions different wheat cultivars were grown in these soils. Wheat cv. Bussard, used in the experiments described in this study, was also the cultivar grown in SSB soil in the field, whereas cv. Monopol was grown successively in the SV soil under field conditions. The influence of the host plant and cultivar on the composition of the microflora is well established for several systems. For example, certain wheat cultivars select for specific populations of *Pseudomonas putida* that are antagonistic to the causal agents of apple replant disease (23), and different tomato genotypes provide better support for the growth and biocontrol activity of *Bacillus cereus* (37). Furthermore, the significance of the plant cultivar has been

TABLE 4. Influence of *Gaeumannomyces graminis* var. *tritici* on wheat root colonization by indigenous fluorescent *Pseudomonas* spp.

Soil	Healthy × diseased roots ^v						Nonlesioned × lesioned root sections of diseased plants ^w					
	Total pseudomonads ^x			<i>phlD</i> + ^y			Total pseudomonads ^x			<i>phlD</i> + ^y		
	– <i>G. graminis</i> var. <i>tritici</i>	+ <i>G. graminis</i> var. <i>tritici</i>	Increase ^z	– <i>G. graminis</i> var. <i>tritici</i>	+ <i>G. graminis</i> var. <i>tritici</i>	Increase ^z	– <i>G. graminis</i> var. <i>tritici</i>	+ <i>G. graminis</i> var. <i>tritici</i>	Increase ^z	– <i>G. graminis</i> var. <i>tritici</i>	+ <i>G. graminis</i> var. <i>tritici</i>	Increase ^z
SV	3.9 × 10 ⁶	3.4 × 10 ⁷ *	8.7	7.6 × 10 ⁵	3.7 × 10 ⁶ *	5.0	5.6 × 10 ⁶	5.6 × 10 ⁷	10.1	4.9 × 10 ⁵	4.8 × 10 ⁶	9.7
SSB	8.1 × 10 ⁶	4.9 × 10 ⁷ *	6.1	7.3 × 10 ⁵	4.3 × 10 ⁶ *	6.0	8.8 × 10 ⁶	3.8 × 10 ⁷ *	4.3	2.7 × 10 ⁵	4.0 × 10 ⁶ *	14.8

^v Mean values of five replicates are presented. Roots of wheat growing in soils nonamended (–*G. graminis* var. *tritici*) or amended (+*G. graminis* var. *tritici*) with *G. graminis* var. *tritici*.

^w Nonlesioned and lesioned root sections of plants infected by *G. graminis* var. *tritici*.

^x Populations of total fluorescent *Pseudomonas* spp. (CFU g^{–1} root).

^y Population of *phlD*+ (CFU g^{–1} root). Means marked with an asterisk (two means in a row) are significantly different according to Wilcoxon's two sample test ($P = 0.05$).

^z Average increase in populations of total fluorescent *Pseudomonas* spp. or *phlD*+ fluorescent *Pseudomonas* spp. in presence of *G. graminis* var. *tritici* (+*G. graminis* var. *tritici*–*G. graminis* var. *tritici*).

TABLE 5. Influence of *Rhizoctonia solani* on wheat root colonization by indigenous fluorescent *Pseudomonas* spp.

Soil	Healthy × diseased roots ^v						Non-lesioned × lesioned root sections of diseased plants ^w					
	Total pseudomonads ^x			<i>phlD</i> + ^y			Total pseudomonads ^x			<i>phlD</i> + ^y		
	– <i>R. solani</i>	+ <i>R. solani</i>	Increase ^z	– <i>R. solani</i>	+ <i>R. solani</i>	Increase ^z	– <i>R. solani</i>	+ <i>R. solani</i>	Increase ^z	– <i>R. solani</i>	+ <i>R. solani</i>	Increase ^z
SV	1.5 × 10 ⁷	1.1 × 10 ⁸ *	7.7	8.5 × 10 ⁵	3.9 × 10 ⁶ *	4.6	8.7 × 10 ⁷	1.4 × 10 ⁸	1.6	5.5 × 10 ⁶	2.3 × 10 ⁶	0.4
SSB	1.7 × 10 ⁷	1.0 × 10 ⁸ *	6.3	1.7 × 10 ⁶	1.1 × 10 ⁷ *	6.6	5.3 × 10 ⁷	1.6 × 10 ⁸ *	3.1	3.0 × 10 ⁶	1.9 × 10 ⁷ *	6.2

^v Mean values of five replicates are presented. Roots of wheat growing in soil nonamended (–*R. solani*) or amended (+*R. solani*) with *R. solani*.

^w Nonlesioned and lesioned root sections of plants infected by *R. solani*.

^x Populations of total fluorescent *Pseudomonas* spp. (CFU g^{–1} root).

^y Population of *phlD*+ (CFU g^{–1} root). Means marked with an asterisk (two means in a row) are significantly different according to Wilcoxon's two sample test ($P = 0.05$).

^z Average increase in populations of total fluorescent *Pseudomonas* spp. or *phlD*+ fluorescent *Pseudomonas* spp. in presence of *R. solani* (+*R. solani*–*R. solani*).

TABLE 6. Influence of *Gaeumannomyces graminis* var. *tritici* on rhizosphere colonization of wheat by introduced *Pseudomonas fluorescens* strains

Isolate	Initial density ^x (CFU per seed)	Final density (CFU g ^{–1} root) ^y		
		– <i>G. graminis</i> var. <i>tritici</i>	+ <i>G. graminis</i> var. <i>tritici</i>	Increase ^z
SSB17	2.1 × 10 ⁴	1.0 × 10 ⁷ a	1.9 × 10 ⁷ a	1.9
SSB17	1.3 × 10 ⁶	1.9 × 10 ⁷ a	3.1 × 10 ⁷ a	1.6
9H4	3.2 × 10 ⁴	2.3 × 10 ⁷ a	2.4 × 10 ⁷ a	1.0
9H4	1.5 × 10 ⁶	1.9 × 10 ⁷ a	2.6 × 10 ⁷ a	1.4
Q8r1-96	2.5 × 10 ⁴	4.7 × 10 ⁷ b	3.7 × 10 ⁷ b	0.8
Q8r1-96	3.9 × 10 ⁶	7.4 × 10 ⁷ b	9.1 × 10 ⁷ b	1.2
4C5	3.9 × 10 ⁴	4.2 × 10 ⁷ b	5.7 × 10 ⁷ b	1.4
4C5	9.3 × 10 ⁶	7.4 × 10 ⁷ b	7.1 × 10 ⁷ b	1.0

^x *Pseudomonas fluorescens* strains SSB17, Q8r1-96, and their respective 2,4-diacetylphloroglucinol-deficient mutants 9H4 and 4C5 were applied to seeds at two different initial densities.

^y Plants were grown in CB soil for 1 month and roots were harvested to determine the final densities in the rhizosphere. Means of five replicates are shown. Means followed by the same letter within columns are not significantly different according to Wilcoxon's two sample test ($P = 0.05$).

^z Increase in population densities of introduced *Pseudomonas fluorescens* strains in presence of *G. graminis* var. *tritici* (+*G. graminis* var. *tritici*–*G. graminis* var. *tritici*).

recently demonstrated at the physiological level. Significant changes in the expression of the *phl* biosynthetic operon of *Pseudomonas fluorescens* strain CHA0 were detected on different maize cultivars (25). Studies using different profiling techniques suggested a significant level of endemism for wheat-associated *phlD*+ fluorescent *Pseudomonas* spp. (40). Given the influence a cultivar may have on the composition and activity of *phlD*+ isolates, the differences between genotypes found in SV and SSB soils as well as between Dutch and U.S. TAD soils may be due to the different wheat cultivars used in different geographical regions and in different fields within a specific region. The effect of wheat cultivars on the genotypic diversity of 2,4-DAPG-producing *Pseudomonas* spp. will be addressed in future studies.

Comparison of the genotypic clustering of the isolates with the clustering based on *in vitro* inhibition of mycelial growth of *G. graminis* var. *tritici* and *R. solani* revealed that this latter approach was less discriminatory. In several host-pathogen systems, antibiotic production by antagonistic microorganisms *in vitro* does not correlate well with the level of suppression of diseases obtained *in situ* (33,44). However, good correlation was found between *in vitro* inhibition and suppression of *G. graminis* var. *tritici* *in situ* by pseudomonads isolated from wheat roots grown in a TAD soil from Washington State (41). In this context, it is interesting to note that the dominant genotypic group found in the Dutch TAD soil, represented by strain SSB17, clustered closely together with Q8r1-96 in the phenotypic groups that were most inhibitory to the take-all fungus. In contrast, SSB17 and Q8r1-96 did not belong to the phenotypic group that was most inhibitory to *R. solani*. More isolates representative of the genotypic and phenotypic groups need to be tested in biocontrol assays to support the earlier results obtained by Weller et al. (41).

Strain SSB17 and strain Q8r1-96, when introduced into the conducive soil (CB) via seed treatment at low densities of approximately 10^3 CFU per seed were able to suppress *G. graminis* var. *tritici* to the same extent as occurred naturally in the suppressive soil (SSB). However, their 2,4-DAPG-deficient mutants 9H4 and 4C5, respectively, did not protect wheat plants against take-all. Although 2,4-DAPG plays a key role in take-all suppression, it does not contribute to the rhizosphere competence of 2,4-DAPG producers, because SSB17 and Q8r1-96 colonized wheat roots to the same extent as their respective 2,4-DAPG-deficient mutants. Similar results were obtained in experiments employing *Pseudomonas fluorescens* strain F113 and its 2,4-DAPG-defective mutant (7). In contrast to 2,4-DAPG, biosynthesis of phenazine antibiotics was shown to significantly contribute to the rhizosphere competence of *Pseudomonas fluorescens* 2-79 and *Pseudomonas aureofaciens* 30-84 (22). Recently, Raaijmakers and Weller (29) demonstrated that Q8r1-96 is much more aggressive as a colonist of wheat roots than other genotypes and it is now known that this unique colonizing ability is shared by strains from other soils that are genotypically similar to strain Q8r1-96. Strain Q8r1-96 was more rhizosphere competent than SSB17 (Fig. 5; Table 6), even though the studies were conducted in raw Dutch soil. This finding supports the suggestion that the rhizosphere competence of Q8r1-96 is minimally affected by the physicochemical characteristics of the soil (29) in which it is growing.

One of the key questions about the phenomenon of TAD is the basis of the enrichment of the antagonist responsible for specific suppression and the effect of the soil environment on the development and expression of suppressiveness. We found no quantitative differences in the population densities of 2,4-DAPG-producing fluorescent pseudomonads on roots of wheat grown in Dutch TAD soils compared with densities reported on roots of wheat grown in Washington State TAD soils (28,30). These findings suggest that the enrichment is independent of the physical and chemical characteristics of the soil. Classical studies of TAD demonstrated that TAD is a field phenomenon requiring the take-all pathogen, a susceptible host, and at least one outbreak of severe disease. Some

early studies suggested that the take-all pathogen, rather than the host plant, is primarily responsible for the selection of specific antagonists (4,6,17,32,36,42,45). For example, Gerlagh (17) and Zogg and Jaggi (45) induced suppressiveness to take-all by repeatedly adding mycelium of *G. graminis* var. *tritici* to soil, but the relationship of this type of suppression to TAD is still not known. In an attempt to begin to address the question about the basis of enrichment of 2,4-DAPG producers in Dutch TAD soils, we compared populations of fluorescent pseudomonads on healthy and diseased roots and on segments of roots from diseased plants with and without lesions. As has been demonstrated many times in the literature (6,39), we found that roots with take-all lesions supported significantly larger populations of fluorescent pseudomonads than healthy roots. Also, 2,4-DAPG producers were enriched on diseased roots but not to a greater extent than the total population of fluorescent *Pseudomonas* spp. Furthermore, no significant increases in population densities of introduced strains SSB17 and Q8r1-96 were observed upon infection of wheat roots by *G. graminis* var. *tritici*. Based upon these limited studies at this time, we conclude that increased nutrient availability upon fungal infection is primarily responsible for the enrichment of 2,4-DAPG producers.

In light of the results of this and earlier studies (28,30), the specificity of the suppression that operates in TAD soils becomes interesting especially when considering the broad-spectrum activity of 2,4-DAPG against many fungal pathogens (20). Australian TAD soils were shown also to have certain levels of suppressiveness to *R. solani*, *Gibberella zeae*, *Pythium irregulare*, *Cochliobolus sativus*, and *Fusarium culmorum* (43). The results of our study, however, indicated that the two Dutch TAD soils were not suppressive to *R. solani*. Given that *R. solani* did not adversely affect population densities of 2,4-DAPG producers (Tables 4 and 5), a possible explanation for a lack of suppressiveness to *R. solani* may be the relative insensitivity of this fungus to 2,4-DAPG (20). Secondly, the 0.5% inoculum rate used in our experiments was high, which may have overwhelmed the system and may explain the failure to detect suppressiveness to *R. solani*. Furthermore, fungi can change patterns of bacterial gene expression (16) and more specifically interfere with the production of 2,4-DAPG (15,25). The suppressiveness of Dutch TAD soils to pathogens other than *G. graminis* and their interaction with 2,4-DAPG-producing *Pseudomonas* spp. are currently under investigation.

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